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Chronic exposure to realistic concentrations of metformin prompts a neurotoxic response in *Danio rerio* adults

Gustavo Axel Elizalde-Velázquez [a], Leobardo Manuel Gómez-Oliván [a,*], Sandra García-Medina [b], Karina Elisa Rosales-Pérez [a], José Manuel Orozco-Hernández [a], Hariz Islas-Flores [a], Marcela Galar-Martínez [b], María Dolores Hernández-Navarro [a]

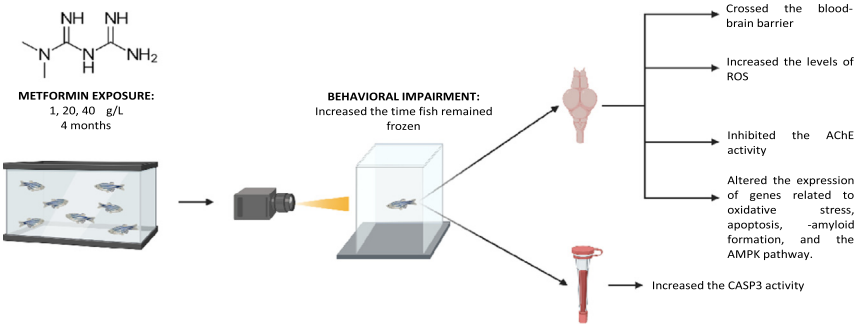
[a] *Laboratorio de Toxicología Ambiental, Facultad de Química, Universidad Autónoma del Estado de México, Paseo Colón intersección Paseo Tollocan, Colonia Residencial Colón, CP 50120 Toluca, Estado de México, Mexico*

[b] *Laboratorio de Toxicología Acuática, Departamento de Farmacia, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Unidad Profesional Adolfo López Mateos, Av. Wilfrido Massieu s/n y cerrada Manuel Stampa, Col. Industrial Vallejo, Ciudad de México CP 07700, Mexico*

HIGHLIGHTS

- Metformin crossed the blood-brain barrier of *Danio rerio* adults.
- Chronic exposure to metformin impaired the swimming behavior of fish.
- Four months of exposure to metformin produced oxidative stress in the brain of fish.
- Metformin promoted the enzymatic activity of *CASP3* in the brain of *Danio rerio*.
- The gene expression of *APP* and *PSEN1* in the brains was upregulated by metformin.

GRAPHICAL ABSTRACT



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ABSTRACT

Metformin (MET) is among the most consumed drugs around the world, and thus, it is considered the uppermost drug in mass discharged into water settings. Nonetheless, data about the deleterious consequences of MET on water organisms are still scarce and require further investigation. Herein, we aimed to establish whether or not chronic exposure to MET (1, 20, and 40 μg/L) may alter the swimming behavior and induce neurotoxicity in *Danio rerio* adults. After 4 months of exposure, MET-exposed fish exhibited less swimming activity when compared to control fish. Moreover, compared with the control group, MET significantly inhibited the activity of AChE and induced oxidative damage in the brain of fish. Concerning gene expression, MET significantly upregulated the expression of *Nrf1*, *Nrf2*, *BAX*, *p53*, *BACE1*, *APP*, *PSEN1*, and downregulated *CASP3* and *CASP9*. Although MET did not overexpress the *CASP3* gene, we saw a meaningful rise in the activity of this enzyme in the blood of fish exposed to MET compared to the control group, which we then confirmed by a high number of apoptotic cells in the TUNEL assay. Our findings demonstrate that chronic exposure to MET may impair fish swimming behavior, making them more vulnerable to predators.

* Corresponding author at: Laboratorio de Toxicología Ambiental, Facultad de Química, Universidad Autónoma del Estado de México, Paseo Colón intersección Paseo Tollocan, Colonia Residencial Colón, CP 50120 Toluca, Estado de México, Mexico
E-mail address: lmgomezo@uaemex.mx (L.M. Gómez-Oliván).

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1. Introduction

Metformin (MET) is the most frequent antidiabetic drug being introduced into the environment. This drug is found in wastewater treatment plants (WWTPs) effluents at proportions of 0.01 to 82 μg/L and in surface waters at quantities of 0.001 to 33.6 μg/L (Meador et al., 2016; Elliott et al., 2017; Yao et al., 2018; Asghar et al., 2018; Posselt et al., 2018; Elizalde-Velázquez & Gómez-Oliván, 2020). However, it is expected that levels of MET in WWTPs effluents and surface waters steadily increase in the upcoming years given its usage for the treatment of cancer and Stein-Leventhal syndrome (Zaidi et al., 2019; Guan et al., 2020).

For MET to be able to induce its beneficial effects, it has to enter the cell through OCT 1 and accumulate in the mitochondria, where it inhibits complex I (Shu et al., 2007; Mallik and Chowdhury, 2018). Once MET inhibits complex I, concomitantly, levels of ATP deplete, and the AMP amount rises, leading to activation of AMPK (Krishan et al., 2015). AMPK activation leads the cell to a catabolic state by cutting off ATP consumption that later switches off glucose, fat, and protein synthesis and increases glucose and fatty acid take up into the cell, generating a cellular energy balance (Mallik and Chowdhury, 2018). Even though AMPK activation appears to have great importance on MET's favorable actions, it also activates different transcription factors associated with cell apoptosis and AB peptide formation, such as p53 and BACE1 (Chen et al., 2009; Li et al., 2015). Moreover, Lee et al. (2019) and Elizalde-Velázquez et al. (2021a) have also associated MET capacity to inhibit mitochondrial complex I with the over-production of ROS. Thus, MET can generate a toxic response in non-target organisms through the same via this drug generates its positive effects.

Upon MET discharge into the surface water, it may induce diverse lethal effects in aquatic organisms. Niemuth et al., 2015 and Crago et al., 2016 for instance, indicated that concentrations of 1 to 100 μg/L of MET induced endocrine disruption in *Pimephales promelas* and in *Danio rerio*, respectively. Furthermore, Ussery et al., 2018 demonstrated that MET (1 to 100 μg/L) significantly altered several metabolites linked to cell metabolism and cellular proliferation and growth in *Oryzias latipes* larvae. Even though the evaluation of behavioral endpoints has become prominent in the last decades, there is still a dearth of knowledge about the effects that some toxicants and drugs induced in the behavior of fish. Inside this list of drugs and toxicants, it is found MET. To date, only three documents that we are conscious of having assessed the impact of MET on the behavior of water organisms. Nonetheless, results are inconclusive, as studies have found different results. Godoy et al., 2018 for instance, pointed out that the swimming behavior of *D. rerio* larvae (120 h post fecundation) was not affected by this drug (0.05–600 mg/L). Similarly, Jacob et al., 2018 established that the distance moved and the swimming velocity of *Salmo trutta f. fario* larvae exposed to MET (1 to 1000 μg/L) were not altered. Nonetheless, in discrepancy with these results, MacLaren et al., 2018 indicated that 40 and 80 μg/L of this drug affected the aggressive behavior of *Betta splendens*. Furthermore, they denoted that this effects persisted after 20 weeks of exposure.

Conduct is a series of actions functioning via the central and peripheral nervous systems (Kane et al., 2005). Alterations in behavior may lead to reduced fitness and survival of organisms and consequently reduced the population level (Brodin et al., 2014). As behavior is not an accidental process, but rather a controlled system of activities meant to guarantee the health and endurance of organisms, behavioral endpoints work as important tools to provide important information on how toxicants impact the health of aquatic organisms.

In the light of this information, the aim of this work was to determine whether MET (1, 20, and 40 μg/L) may alter the swimming behavior of *D. rerio* adults, after 4 months of exposure. Furthermore, oxidative stress biomarkers in the brain and the activity acetylcholinesterase (AChE) of fish exposed to this drug were also evaluated. We hypothesize that metformin will cross the blood-brain barrier of fish, triggering an oxidative stress response, which in turn will prompt a disruption in their swimming behavior.

2. Method

2.1. Reagents

All reagents, but Metformin hydrochloride (MET, CAS number: 1115-70-4), were procured from Sigma-Aldrich (St. Louis, MO). The latter was acquired from Toronto Research Chemicals (Toronto, ON).

2.2. Housing systems

Five-month-old female and male zebrafish adults (AB strain) were allocated to different systems, each with 50 fish (50 L), provided with tap water treated with charcoal and UV light. Table 1 describes all systems parameters and conditions we took care of during zebrafish housing and exposure. Feeding was done three times/day with Spirulina flakes (Ocean Nutrition, US).

2.3. Exposure systems

250 *D. rerio* grown-ups were allotted to six tanks of 50 L of capacity. Four of the six systems contained 50 fish; while, the remaining two had 25 fish (≈1 male:1 female ratio). Every system with 50 fish was allocated one of the three MET treatment concentrations (1, 20, and 40 μg/L), with one system assigned as the control treatment. The same unchanged procedure was carried out 3 times in 3 independent tests. Systems with 25 fish, on the other hand, were used as positive controls for acetylcholinesterase (10 μg/L of Atrazine) and oxidative stress (3 μg/L of Atrazine). MET concentrations used in this experiment span concentrations that have been demonstrated to prompt oxidative damage in *D. rerio* (Elizalde-Velázquez et al., 2021a, 2021b) and extended to higher concentrations that have been shown to induce behavioral changes in *Betta splendens* (MacLaren et al., 2018). Moreover, for atrazine, we chose these concentrations because previous studies have reported that at those concentrations, this herbicide induced oxidative damage, increased the levels of antioxidant enzymes, and inhibited acetylcholinesterase in fish (Jin et al., 2010; Nwani et al., 2010; Xing et al., 2012; Blahová et al., 2013; Schmidel et al., 2014; Liu et al., 2016). Renewal of the medium from each system was done every other day through all exposure. Moreover, we daily removed and counted dead organisms from each aquarium to then calculate the LC_{50}.

2.4. Behavioral assessment

A mean of 32 fish (S.D. ± 2) per group of MET treatment survived after four months of exposure. However, we only selected 20 of them, on a random basis, to be used for the evaluation of behavior. The Novel Tank Test explained by Elizalde-Velázquez et al. (2022) was used to assess the swimming activity of fish. Briefly, 20 randomly selected fish per concentration of MET were moved to and kept in the experimental room for a minimum of 50 min. Next, fish were individually selected and placed into a 15 L rectangular novel tank for 12 min (2 min of acclimatization, 10 min of trial). During the trial time, a tape of each *Danio rerio* adult was recorded and analyzed with Tox Track Ink software. For biases to be avoided in the Novel Tank test, fish were always transferred from the husbandry room to the behavioral room at 7 am. Moreover, behavioral tests were only performed from 8 am to 10 am and on several days. To avoid experiments lasting too many days, we opted to record videos from different fish in a

Table 1
Systems parameters and conditions.

Parameters	Measured value
T°	27 ± 1 °C
Light:Dark cycles	14 h:10 h
Dissolved oxygen	9.0 ± 0.4 mg/L
NO_2^-	0.026 ± 0.005 mg/L
NO_3^-	2.3 ± 0.2 mg/L
pH	7.32 ± 0.15

simultaneous manner. Novel Tank test was performed three times in three independent experiments, and for each experiment, we used a total of 20 organisms per group of treatment ($n = 3$).

2.5. Fish blood and brain collection

After the behavioral assessment, we aimed to collect the blood and brains of all fish from all treatment groups. Accordingly, we extracted the brains and collected the blood of a mean of 32 fish (S.D. ± 2) per group of MET treatment. For blood collection, we anesthetized all fish with an MS-222 solution (150 mg/L) and followed the protocols described by Babaei et al., 2013. Briefly, after cutting the caudal fin of an anesthetized fish, we formed an assembly that consisted of the fish, with the wound directed down, a punctured tube, and an additional Eppendorf tube. Each assembly was then centrifuged at 40*g* for 5 min at 11 °C. The blood gathered was employed for the assessment of apoptosis. For each of the three experiments, we collected a mean of 11 μL (S.D. ± 2 μL) of blood per fish, resulting in a total of 350 μL (S.D ± 30 μL) of blood per treatment group.

Following blood collection, fish from all treatment groups were euthanized using the hypothermic shock method (2–4 °C). When fish did not show any signs of life, we excerpt the brains of all fish. Extracted brains were then allocated in Eppendorf tubes with 1 mL of PBS (pH 7.4). For each concentration of MET, including the control group, we got four Eppendorf tubes, each with eight brains, that we used for each of the following biomarkers: oxidative stress, acetylcholinesterase activity, and gene expression. Moreover, one of these tubes was also used to determine the presence of MET in the brain of fish.

2.6. Determination of oxidative stress biomarkers

Employing an Ultra-turrax T25 (IKA, Germany), brain samples were homogenized and then allocated into two Eppendorf tubes. Tube 2 comprised only 700 μL of the homogenate, and tube 1 encompassed 300 μL of sample and 300 μL of TCA. Tubes 1 and 2 were centrifuged at 11495 and 12,500 rpm, and content was used to assess all oxidative stress biomarkers (Table 2). All results were normalized against total proteins. For the assessment of oxidative stress biomarkers and subsequent brain endpoints, we analyzed the brain samples of the three independent experiments. Moreover, for all biomarkers, we evaluated samples three times. Thus, we got a total of nine results per biomarker and per treatment group ($n = 9$).

2.7. Acetylcholinesterase (AChE) assessment

AChE activity was ascertained by using the method established by Ellman et al., 1961. Briefly, *D. rerio* brains were homogenized and centrifuged at 10000 rpm for 15 min. Afterward, using glass test tubes, 400 μL of the supernatant was mixed with 2.6 mL of PBS (pH 7.4), 0.1 mL of DTNB (5,5-dithiobis-2-nitrobenzoate, 0.1 M), and 25 μL of substrate (acetylthiocholine iodide 0.075 M). Variations in absorbance were quantified at 412 nm and documented every minute for 5 min.

2.8. qRT-PCR

Upon RNA isolation (RNeasy® kit of Qiagen), we ascertained its concentration and purity via 260/280 ratio and agarose (1 %) gel electrophoresis. 1 μg of the total RNA was employed to carry out reverse transcription reactions (QuantiTect® Reverse Transcription Kit) under the following conditions: 42 °C for 15 min and 95 °C for 3 min. cDNA was used as a template for qRT-PCR, which was carried out in Rotor-Gene Q and under the following conditions: 94 °C for 15 s, followed by 35 cycles of 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. All samples were normalized against B-actin (housekeeping). mRNA expression changes were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Every PCR reaction was repeated three times to minimize experimental error (see Table 3).

2.9. CASP3 activity

Jurkat cells (ATCC TIB-152) at a concentration of 10^6 cells/mL were cultured with 50 ng/mL of anti-Fas mAB (clone CH-11, MBL international) for positive control of apoptosis. Moreover, for the negative control of apoptosis, Jurkat cells were cultivated with 125 L of a solution 20 mM of Z-VAD-FMK. Both cultures were incubated at dusk (16 h at 37 °C) in a wet atmosphere with 5 % CO_2. To obtain the cell extracts, we followed the protocol established by Orozco-Hernández et al., 2019. Briefly, the above cultures were centrifuged at 450 ×*g* for 10 min, and the gotten pellets were then rinsed with PBS at 4 °C and re-dissolved in a lysis buffer (10^8 cells/mL). The cells were lysed by a freezing-thawing process and incubated on ice for 15 min. Upon cells were lysed, we centrifuged them again at 15,000 ×*g* at 4 °C for 20 min, and the supernatant was used in the test.

Once we got the cell extracts, we performed the colorimetric assay according to the established protocols of the kit CaspASE, Promega. Briefly, by using microplates with a flat bottom, we prepared the below systems:

- Blank system: It contained 32 μL of the caspase buffer, 2 μL DMSO, 10 μL dithiothreitol (DTT, 100 mM), 54 μL of deionized water, and 2 μ of substrate DEVD-pNA.
- MET systems: It contained the same reagents as the blank system plus 20 μL of a blood sample of fish from each MET treatment.
- Positive and negative systems: It contained the same reagents as the blank system plus 20 μL of the positive or negative cell extract, respectively.

Table 2
Methods used for oxidative stress biomarkers determination.

	Biomarker	Sample amount	Reagents	Wavelengths	Method used
Tube 1	LPX	50 μL supernatant	450 μL Tris-HCl 150 nM 1 mL TCA-TBA	535 nm	Buege and Aust, 1978
	HPX	100 μL supernatant	900 μL mixture ($FeSO_4$, H_2SO_4, dehydroxytoluene butylate, and xylenol orange).	560 nm	Jiang et al., 1992
	POX	Precipitate	150 μL DNPH/HCl 10 nM 500 μL TCA 1 mL guanidine 6 M	366 nm	Levine et al., 1994
Tube 2	SOD	40 μL supernatant	260 μL CO_3 buffer (50 mM Na_2CO_3 and 0.1 mM EDTA) 200 μL adrenaline 30 mM	480 nm	Misra and Fridovich, 1972
	GPX	100 μL supernatant	290 μL reaction buffer (3.5 mM GSH, 1 mM NaN_3, and 0.12 mM NADPH) 100 μL H_2O_2 20 mM 12 μL GR	340 nm	Gunzler and Flohe-Clairborne, 1985
	CAT	30 μL supernatant	420 μL isolation buffer (0.3 M sucrose, 1 mM EDTA, 5 mM HEPES, and 5 mM KH_2PO_4) 300 μL H_2O_2 20 mM	240 nm	Radi et al., 1991
	TP	13 μL supernatant	300 μL distilled water 1.25 mL Bradford reagent (Coomassie blue, Et-OH 96 %, H_3PO_4).	595 nm	Bradford, 1976

Table 3
Sequence of genes tested.

Gene	Forward primer	Reverse primer	Reference
Nrf1	TTT GGT TCC CGA TGA AGA CG	TGA TTA GCG TGA GAC TGA GC	Timme-Laragy et al., 2012
Nrf2	ACC CAA TAG ATC TAC AGA GC	GGT GTT TGG ACA TCA TCT CG	Timme-Laragy et al., 2012
BAX	GGC TAT TTC AAC CAG GGT TCC	TGC GAA TCA CCA ATG CTG T	Soares et al., 2017
CASP3	CCG CTG CCC ATC ACT A	ATC CTT TCA CGA CCA TCT	Félix et al., 2018
CASP 9	CGG AGG AGG TGA GAA GGA TAT	TCC AGC ACA CGA TCA AGA TT	Jiang et al., 2014
p53	GCA GCG ATG AGG AGA TCT TT	GGG CTC AGA TGA TTC ACG AT	Lei et al., 2017
PRKAA1	TGT GAG GAC GCA GCA AAA GG	GAG GTA AGA GAA GAG GCC AG	Zang et al., 2019
PRKAA2	CGT CAA GAA GGC AAA GTG GC	TTC TTC CGG CGC ACT CTT AG	Zang et al., 2019
BACE1	TTA CAT AGA GAT GGC GGT GGG	GAG GAG AGT GAG CGG TGG TAA TA	Moussavi Nik et al., 2012
APP	GGT GGA GGT GCC GTC AGA	GGT GGA GGT GCC GTC AGA	Moussavi Nik et al., 2012
PSEN1	CTC TGC ACT CCA TGC TCA ACG	CAG CCA GGC TTG AAT CAC CTT	Moussavi Nik et al., 2012

Nrf1 = Nuclear Respiratory Factor 1. ***Nrf2*** = Nuclear Respiratory Factor 2. ***BAX*** = BCL2 Associated X Protein. ***CASP3*** = Caspase 3. ***PRKAA2*** = Protein Kinase AMP-Activated Catalytic Subunit α 2. ***PRKAA1*** = Protein Kinase AMP-Activated Catalytic Subunit α 1. ***APP*** = Amyloid β Precursor Protein. ***GRID2IP*** = Glutamate Receptor, Ionotropic, δ 2-Interacting Protein 1. ***PCDH17*** = Protocadherin 17. ***PCDH19*** = Protocadherin 19.

Finally, systems were incubated at 37 °C for 4 h, and then we read absorbance at 405 nm. In the case of the CASP3 activity and TUNEL tests, we analyze blood samples only once as the volume of blood collected reached to perform one replicate. However, as mentioned above, we performed three independent experiments; so, we got three different results for each test ($n = 3$). Data collected was then used to perform statistical analyses and bar charts.

2.10. TUNEL assay

To identify apoptotic cells, we used the ApopTag Fluorescein S7110 kit (Chemicon), which consists of 5 sequential steps:

1) Lymphocytes obtainment: For this purpose, 300 μL of blood plasma from fish exposed to each MET treatment were centrifuged at 3000 ×*g* for 5 min at 4 °C. Once we got lymphocytes, we resuspended them with 50 μL of mounting solution
2) Cellular fixation: We placed 1 μL of the above solution on a slide previously prepared with poly-L-lysine, and then we dried it at 60 °C for 5 min. Following this, we fixed the cell material to the slides by adding cold acetone for 10 min.
3) Hydration: The above-prepared slides were placed in several solutions of xylene-alcohol at 96, 80, 70, 60, and 50 % (v/v) for 30 s. Following this, we added 1 μL of proteinase k (20 mg/mL) to hydrated cells, and then we washed them three times with PBS at a pH 7.4. After washings, we added 60 μL of the equilibrium buffer and 65 μL of the TdT enzyme to the slides and incubated them at 37 °C for 1 h.
4) Cell labeling: We added the anti-FITC conjugate to the slides for 30 min.
5) Cell staining: A propidium iodide solution (1.5 μg/mL) was used to dye the cells.

For negative control, we used the protocol described above, but without the addition of TdT. Moreover, for positive control, we used cells treated with 1 mg/mL of DNase. A hundred cells were analyzed per blood sample.

2.11. Glucose quantification

To avoid disturbances in behavior results, we opted to use other fish to determine levels of glucose. Consequently, four systems, 20 *D. rerio* each, were assigned to aquaria of 20 L of capacity. Each system was allocated one of the three metformin treatments (0, 1, 20, 40 μg/L). These fish were treated under the same conditions as the fish mentioned in Sections 2.3 and 2.4.

For glucose determination, we used the method reported by Zang et al., 2015. Briefly, each week along the four months of exposure, we selected a pool of 5 fish from each system and anesthetized them with 2-phenoxyethanol (500 ppm). Once fish were anesthetized, we inserted a heparinized needle near the caudal fin of the fish. Next, with the help of a bulb dispenser, we expelled the blood onto a clean area of a piece of parafilm, and then we measured the glucose levels using a commercial handheld glucometer (Accu-Chek Aviva meter, US). Quantification of blood glucose was performed on each of the fish individually. At the end of the glucose quantification test, we got 80 results per group of treatment, as we measured blood glucose from 5 fish of each MET concentration, including the control group, per week. Thus, we opted to perform this experiment once and carry statistical analyses and bar charts out with obtained data ($n = 320$).

2.12. Metformin determination in the brain of zebrafish

MET concentrations were determined in water and brains. Water samples (10 mL) from each of the systems were collected every week during the four months of exposure. Brain samples from three independent experiments were treated following the protocol described by Łabuzek et al., 2010. Concisely, the homogenate was deproteinized (acetonitrile and MeOH added with internal standard), sieved (10 μm strainer), and evaporated to dryness (N_2 stream). Next, samples were dissolved with 100 μL of mobile phase and centrifuged at 16000 *g* for 15 min. 50 μL of supernatant was injected onto the autosampler. Quantification was performed with the use of an Agilent 1260 HPLC system coupled to an API 5500 Qtrap MS with a Turbo V Ion spray source. Nitrogen (>99 %) was used as desolvation spray and collision gas. Separation was achieved employing an Xbridge C18 column (50 mm × 3.0 mm, particle size 3.5 μm). The mobile phase consisted of 2 mM acetate buffer in water as eluent A and 100 % acetonitrile as mobile phase B. The samples were eluted at a flow rate of 100 μL/min. Data were evaluated with Analyst 1.6 software.

2.13. Statistical analysis

Results from all tests were uttered as the mean ± standard deviation (SD). To determine the differences between means, we used a Tukey test, considering a $p < 0.05$. Moreover, a one-way ANOVA test ($\alpha = 0.05$) was applied to ascertain the differences between treatment groups (Sigma Plot 12.3). Normality and homoscedasticity were checked by Shapiro-Wilk and Bartlett tests, respectively. We carried out a principal component analysis to correlate all variables measured (R software; $p < 0.05$).

3. Results

3.1. Mortality

After four months of exposure, MET generated the death of several adult zebrafish. It is noteworthy that more deaths occurred in the last two months of exposure (Fig. 1). From Fig. 1, it also can be appreciated that the mortality rate of MET increased in a concentration-dependent manner. Nonetheless, we did not find significant differences between treatment groups.

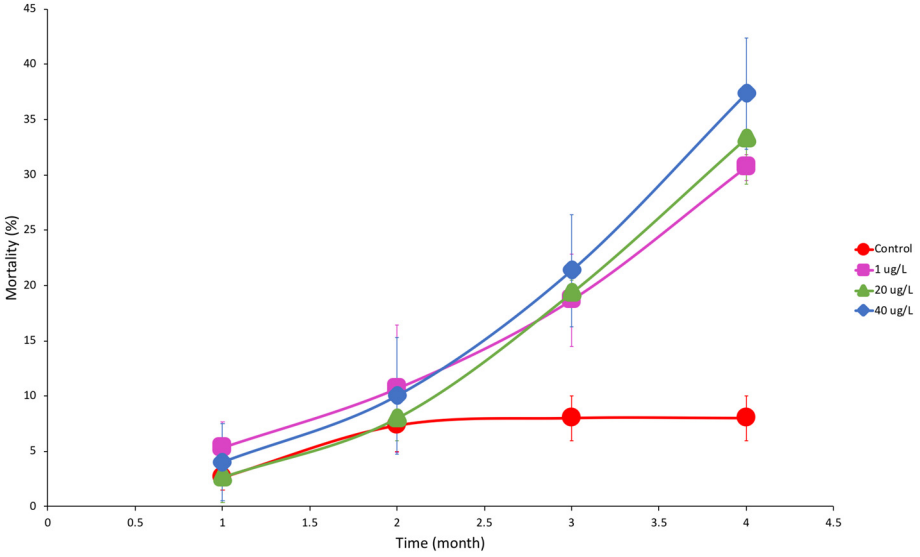


Fig. 1. Mortality of zebrafish adults exposed to each MET concentration at 1, 2, 3, and 4 months. Each point represents the mean ± SD, $n = 3$.

3.2. Behavioral assessment

According to our results, MET induced changes in the behavior of *D. rerio* adults. For example, MET significantly reduced the total distance traveled ($F(3,236) = 118.334$; $p < 0.001$) (Fig. 2A), the distance traveled in the top ($F(3,236) = 612.311$; $p < 0.001$) (Fig. 2B), and time spent in bottom ($F(3,236) = 37.647$; $p < 0.001$) (Fig. 2C) in all fish compared to the control group. The decreases we observed in all these endpoints were in a concentration-dependent manner. Accordingly, we observed significant differences between treatment groups. Concerning distance traveled in bottom ($F(3,236) = 66.212$; $p < 0.001$) (Fig. 2B), time spent in top ($F(3,236) = 39.044$; $p < 0.001$) (Fig. 2C), and the time frozen in the top ($F(3,236) = 1449.755$; $p < 0.001$) and bottom ($F(3,236) = 51.655$; $p < 0.001$) (Fig. 2D), fish exposed to MET showed a significant increase compared to the control group. It is noteworthy to say that as concentration increased, the value of these endpoints also increased. Hence, we observed significant differences between treatment groups for all these biomarkers, except for the frozen in the bottom.

3.3. Oxidative stress

According to our data, the enzymatic activity of SOD ($F(4,40) = 80.160$; $p < 0.001$) (Fig. 3A), CAT ($F(4,40) = 92.458$; $p < 0.001$) (Fig. 3B), and GPX ($F(4,40) = 172.668$; $p < 0.001$) (Fig. 3C) in all MET treatment groups and positive control group showed a significant increase in comparison to the control group. Moreover, similar to the activity of antioxidant enzymes, levels of all oxidative damage biomarkers in all treatment groups significantly increased in relation to the control group (LPX ($F(4,40) = 71.373$; $p < 0.001$) (Fig. 3D), HPX ($F(4,40) = 115.582$; $p < 0.001$) (Fig. 3E), POX ($F(4,40) = 110.416$; $p < 0.001$) (Fig. 3F). Accordingly, MET and atrazine induced an oxidative stress response in the brain of fish. However, unlike MET, atrazine induced a more severe response. For example, from Fig. 3A–F, it can be seen that for all biomarkers, positive control values were twice above the value of the control group. Meanwhile, levels of biomarkers on fish exposed to MET increased as the concentration also did, but these never overpassed values of positive control. Even though the values of MET never overpassed the value of positive control, it is noteworthy to mention that we did not find significant differences between the positive control and the highest concentration of MET in HPX, POX, and CAT.

3.4. AChE activity

AChE activity alterations in the brain of *D. rerio* adults are depicted in Fig. 4. From this figure, we can observe that a concentration of 1 μg/L, MET induces a significant increase in the activity of AChE compared to the control group ($F(4,40) = 620.931$; $p < 0.001$). Nonetheless, as concentration increase, MET inhibit the activity of this enzyme. At concentrations of 20 μg/L and 40 μg/L, for instance, we observed that this drug significantly reduced the activity of AChE compared to the control group ($F(4,40) = 620.931$; $p < 0.001$). Concerning positive control, we can see that there is a significant difference between MET treatments and this. Moreover, as changes in AChE activity in the brain of *D. rerio* occurred in a concentration-dependent manner, we observed significant differences between MET treatment groups as well.

3.5. qRT-PCR

MET significantly increased the gene expression of *Nrf1* ($F(3,32) = 766.541$; $p < 0.001$) and *Nrf2* ($F(3,32) = 841.688$; $p < 0.001$) compared to the control group (Fig. 5). The increase observed in these two genes was in a concentration-dependent manner. Between these two genes, we observed a higher increase in the gene expression of *Nrf2* compared to *Nrf1*. Concerning apoptosis-related genes, we saw a significant decrease in the expression of *CASP3* ($F(3,32) = 42.059$; $p < 0.001$) and *CASP 9* ($F(3,32) = 42.319$; $p < 0.001$) compared to the control group (Fig. 5). Nonetheless, the gene expression of *BAX* ($F(3,32) = 390.728$; $p < 0.001$) and *p53* ($F(3,32) = 506.137$; $p < 0.001$) increased in a concentration-dependent manner compared to the control group. *p53* was more overexpressed than *BAX*, and *CASP3* was more downregulated than *CAPS9*. Interestingly, we also observed a significant increase in the gene expression of *BACE1* ($F(3,32) = 898.793$; $p < 0.001$), *APP* ($F(3,32) = 884.977$; $p < 0.001$), and *PSEN1* ($F(3,32) = 447.258$; $p < 0.001$) at all concentrations of MET compared to the control group (Fig. 5). Among these genes, the more overexpressed was *APP*, followed by *BACE1* and *PSEN1*, respectively. Finally, compared to the control group, we saw a significant increase in the expression of *PRKAA1* ($F(3,32) = 543.169$; $p < 0.001$) and *PRKAA2* ($F(3,32) = 1001.103$; $p < 0.001$) as well (Fig. 5). *PRKAA2* was more overexpressed than *PRKAA1*.

3.6. CASP3 activity

Intriguingly, though MET did not overexpress the CASP3 gene, we saw a significant increase in the activity of this enzyme in the blood of fish exposed to MET compared to the control group, except for the group exposed to the lowest concentration of this drug ($F(5,12) = 162.572$; $p < 0.001$) (Fig. 6). The increases we observed in CASP3 enzymatic activity were in a concentration-dependent manner. Thus, we observed significant differences between MET treatment groups. Similar to the control group, we also observed CASP3 activity in all treatment groups increased compared to the negative control. Nonetheless, regarding positive control, we observed CASP3 activity in all treatments significantly reduced compared to this.

3.7. TUNEL assay

As we witnessed MET induced the activity of CASP3 in the blood of *D. rerio* adults, we opted to quantify the number of apoptotic cells via TUNEL assay. According to our results, we saw MET increased the number of apoptotic cells in a concentration-dependent manner (Fig. 7). Thus, we found the highest number of apoptotic cells at the concentration of 40 μg/L ($F(5,12) = 251.045$; $p < 0.001$). Unlike positive control, which induced 67 % of cell death in the blood of *D. rerio*, MET generated 22.3 31.4, and 37.6 % of cell death at concentrations of 1, 20, and 40 μg/L, respectively.

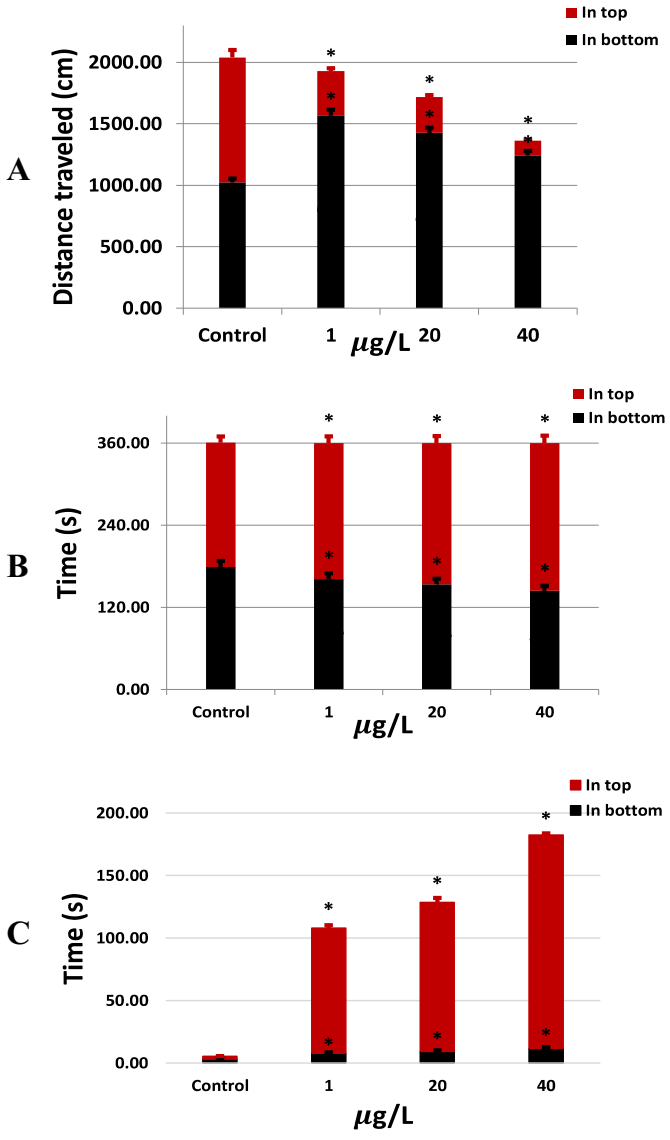


Fig. 2. Behavioral endpoints (A: distance traveled in the top and bottom, B: time spent in the top and bottom, C: time frozen) evaluated in *D. rerio* adults exposed to MET. Data were represented as mean ± SD, $n = 3$. * denotes a significant change in comparison to the control group.

3.8. Metformin determination in the brain of zebrafish

All measured concentrations of MET in the brain of zebrafish were above the limit of quantification (Table 4). Thus, MET was able to cross the blood-brain barrier and had an impact on the brain of zebrafish. From Table 4, we can see that as the concentration of MET increased in the water of exposure, the quantity of this drug in the brain of fish augmented as well. Thus, we found the highest concentration of MET in the brain of fish exposed at the concentration of 40 μg/L.

Concerning water samples, MET concentrations in the control group remained <LOQ; meanwhile, measured concentrations of MET in the remaining treatment groups decreased, but no >20 % compared to the control group (Table 5).

3.9. Glucose levels

As behavioral changes observed in this study could be related to the MET capacity to decrease glucose levels in the blood, we opted to evaluate this biomarker in fish.

From, Fig. 8, we can observe that after chronic exposure to MET, glucose levels of fish decreased in a concentration-dependent manner ($F(3,316) = 994.258$; $p < 0.001$). Thus, we observed significant differences between treatment groups and the control group and between all treatment groups.

3.10. Principal component analysis

According to our principal component analysis, almost all biomarkers tested herein are strongly correlated. For example, from Fig. 9, we can see there is a positive correlation between oxidative stress biomarkers and the gene expression of *Nrf1* and *Nrf2*. Moreover, oxidative stress biomarkers are also positively correlated to expression B-amyloid formation-, and apoptosis- related genes, except for *CASP3*. Unlike the gene expression of *CASP3*, which is poorly correlated to the rest of the genes, the enzymatic activity of *CASP3* is strongly correlated to oxidative stress biomarkers and the gene expression of *p53* and *BAX*. The above is noteworthy because our TUNEL assay results showed a good correlation with the enzymatic activity of *CASP 3*.

4. Discussion

MET is among the most consumed drugs in the world, and due to this reason, it is also one of the highest drugs by weight released into the aquatic environments. Recently, this drug has been found in WWTPs effluents at concentrations of 0.010 g/L to 82.7 g/L and in surface waters at concentrations of 0.0002 g/L to 33.6 g/L (Meador et al., 2016; Elliott et al., 2017; Yao et al., 2018; Asghar et al., 2018; Posselt et al., 2018; Elizalde-Velázquez & Gómez-Oliván, 2020). Nonetheless, as MET consumption is expected to increase due to rising evidence of its usage in non-diabetic indications, levels of this drug in aquatic environments are also likely to increase. Up to date, number of evidence have pointed out this drug can generate endocrine disruption, oxidative stress, and embryotoxicity on different water species. However, there is still a knowledge gap about the negative behavioral impact this drug may cause in fish. The present study aimed to investigate whether chronic exposure to MET (1, 20, and 40 μg/L) may disrupt *D. rerio* adults' swimming behavior and induce toxic effects in the brains of this freshwater organism. Our findings demonstrated that MET-exposed fish exhibited less swimming activity when compared to control fish. For example, our results showed fish exposed to MET exhibited a significant reduction in the total distance traveled due to an increase in the

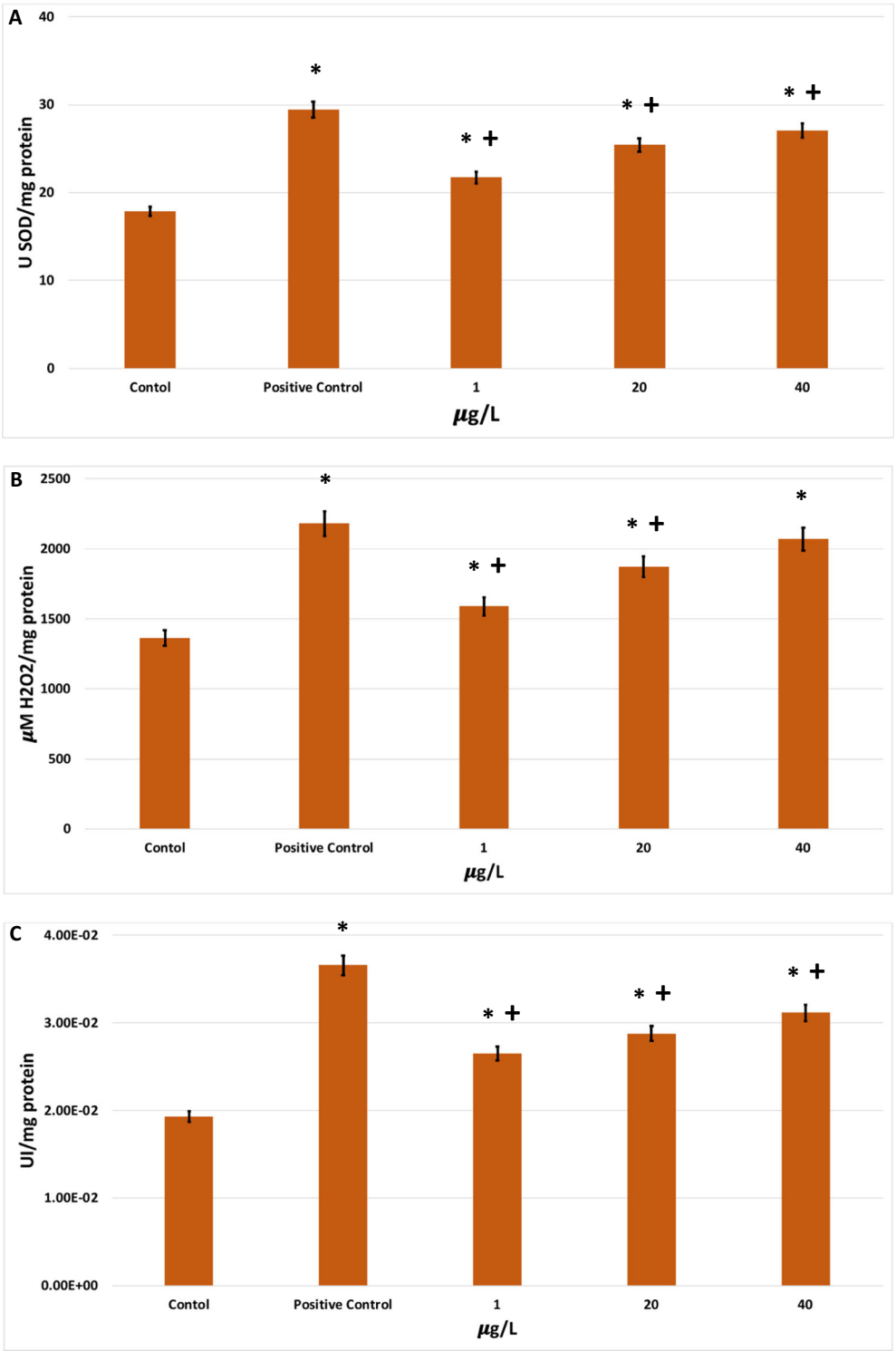
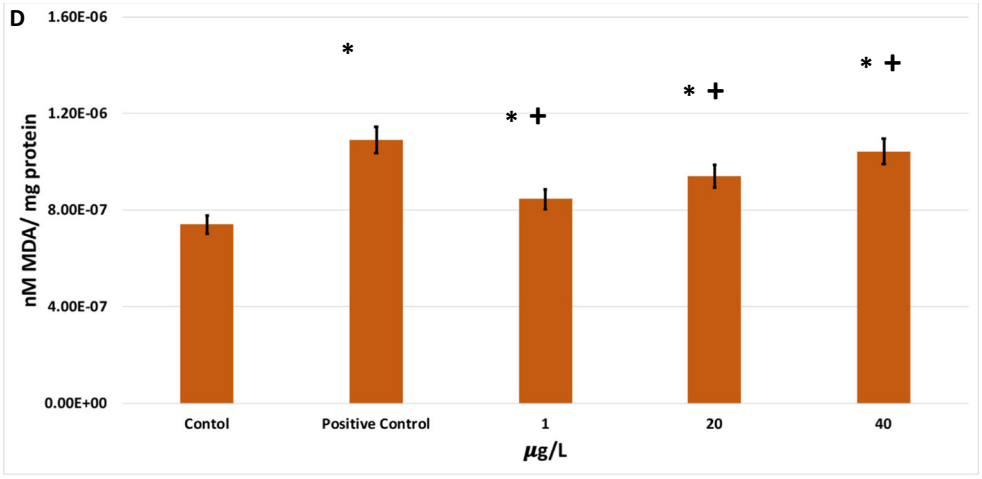
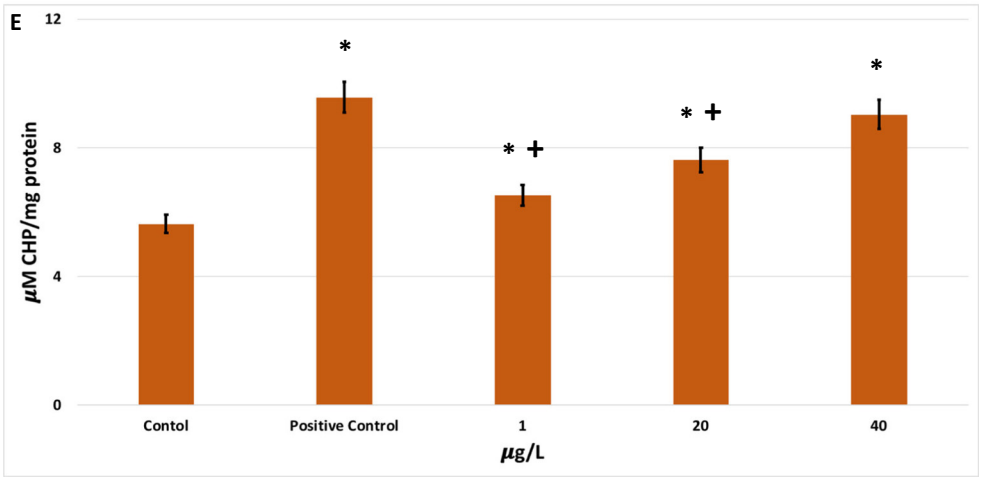


Fig. 3. Brain levels of A: SOD, B: CAT, C: GPx, D: LPX, E: HPX, and F: POX in *D. rerio* adults exposed to MET. Data were represented as mean ± SD, $n = 9$. * denotes a significant change in comparison to the control group. + denotes a significant change in comparison to the positive control.

time they remained frozen. In agreement with our results, MacLaren et al., 2018 demonstrated that *Betta splendens* adults exposed to MET (40 and 80 μg/L) exhibited a significant reduction in aggressive behavior after 4 and 20 weeks of exposure. Moreover, other studies carried out in zebrafish larvae have also demonstrated that MET may alter their locomotor and cognitive function. For example, Phillips et al., 2021 reported that five days of exposure to MET (0.16, 165.62, and 1656.2 μg/L) led to hypoactivity during dark periods in zebrafish larvae. Thus, organisms exposed to this drug may experience different behavioral changes, even at low doses/concentrations.

Neurotoxic effects induced by MET may be closely related to its mechanism of action. As it is well well-known, one of the MET's functions is the





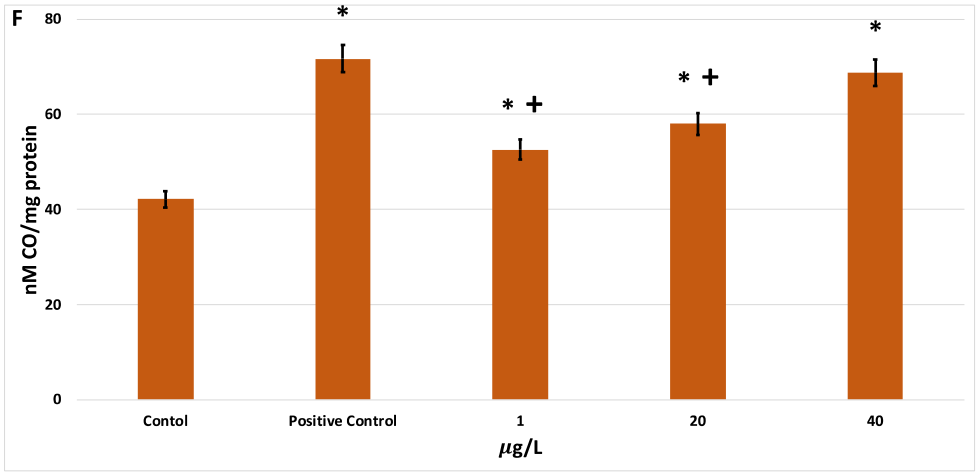


Fig. 3 (*continued*).

inhibition of mitochondrial complex I, which leads to diverse alterations in the mitochondrial structure and ATP production (Andrzejewski et al., 2014; Cameron et al., 2018). Inhibition of mitochondrial complex I is also related to the generation of ROS. Lee et al., 2019 for instance, pointed out that the inhibition of mitochondrial complex I by MET could disturb the electron stream and cause the superoxide generation by a flavin mononucleotide reduction. ROS are important in redox signaling from the organelle to the rest of the cell. Nonetheless, during oxidative stress, ROS may lead to cell death by stimulating the generation of MAP kinases and nuclear transcription factors (*NF-κB*, *AP-1*, and *p53*) (Reuter et al., 2010). Due to its limited antioxidant system and its high content of polyunsaturated fatty acids, the brain is particularly vulnerable to the damaging effect of ROS (Fatma et al., 2018). To our knowledge, this is the first study that demonstrates MET may induce oxidative stress in the brain of fish. In agreement with these results, Gou et al., 2021 demonstrated MET induced the accumulation of ROS in human hepatocellular carcinoma HepG2 cells by inhibiting the expression of *Nrf1*. Intriguingly, unlike these two studies, our results showed MET upregulated the expression of *Nrf1* and *Nrf2* in the brain of zebrafish. The above could be due to the antioxidant mechanism of MET. Previous studies have demonstrated MET may protect different cell cultures

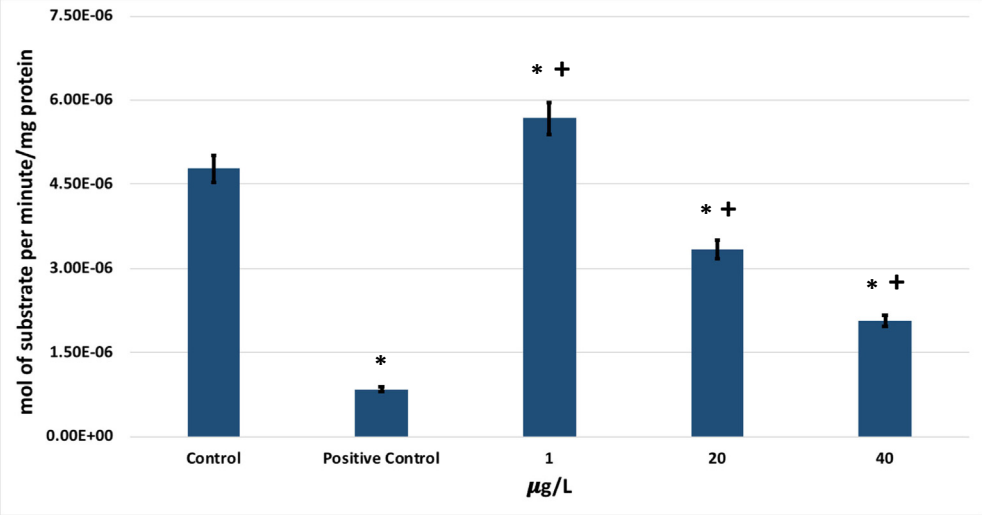


Fig. 4. AChE activity in the brain of *D. rerio* adults exposed to MET. Data were represented as mean ± SD, $n = 9$. * denotes a significant change in comparison to the control group. + denotes a significant change in comparison to the positive control.

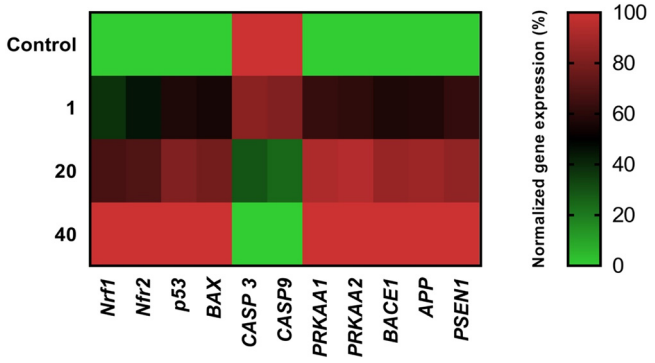


Fig. 5. Chronic exposure to MET alters different genes related to oxidative stress (*Nrf1* and *Nrf2*), apoptosis (*CASP3*, *CASP9*, *p53*, and *BAX*), metformin signaling pathway (*PRKAA1* and *PRKAA2*), and β amyloid formation (*BACE1*, *APP*, and *PSEN1*) in the zebrafish brain. Data were represented as mean ± SD, $n = 9$. *: significant difference compared to the control group.

from oxidative stress due to its capacity to activate the *Nrf2* transcription factor via AMPK signaling (Ashabi et al., 2015; Arbab et al., 2021). Nonetheless, like us, other studies have also indicated MET's lack of antioxidant effect, and instead, have shown this drug to induce a toxic response in different organisms and cells (Allard et al., 2016; Ussery et al., 2018; Lee et al., 2019; Lin et al., 2020; Elizalde-Velázquez et al., 2021a, 2021b). Consequently, *Nrf1* and *Nrf2* genes activation could be related to the increased production of ROS by MET. Gureev et al., 2019, for instance, pointed out ROS, particularly H_2O_2, are strong *Nrf2* activators.

ROS overproduction and Nrf1 and Nrf2 alterations above mentioned may create a vulnerable environment in the brain, leading to different alterations in their structure and function (Allard et al., 2016). One of these brain function alterations could be the inhibition of AChE activity. Herein, for instance, we demonstrated that the AChE activity in the brain of fish exposed to 1 µg/L of MET increased compared to the control group. Nonetheless, at higher concentrations (20 and 40 µg/L), MET significantly inhibited the activity of this enzyme in fish. Oxidative stress may be implicated in the inhibition of AchE. Molochkina et al., 2005 for instance, showed that H_2O_2 modifies AChE activity via shifts it induces in the structure of lipid bilayer. In addition, Schallreuter and Elwary (2007), found that H_2O_2 controls

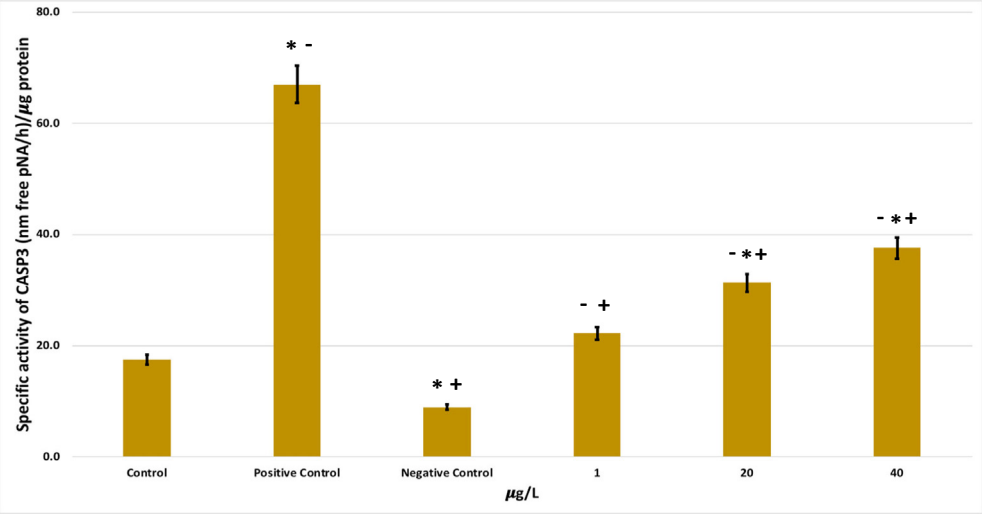


Fig. 6. CASP3 activity in the blood of *D. rerio* adults exposed to MET. Data were represented as mean ± SD, $n = 3$. * denotes a significant change in comparison to the control group. + denotes a significant change in comparison to the positive control. - denotes a significant change in comparison to the negative control.

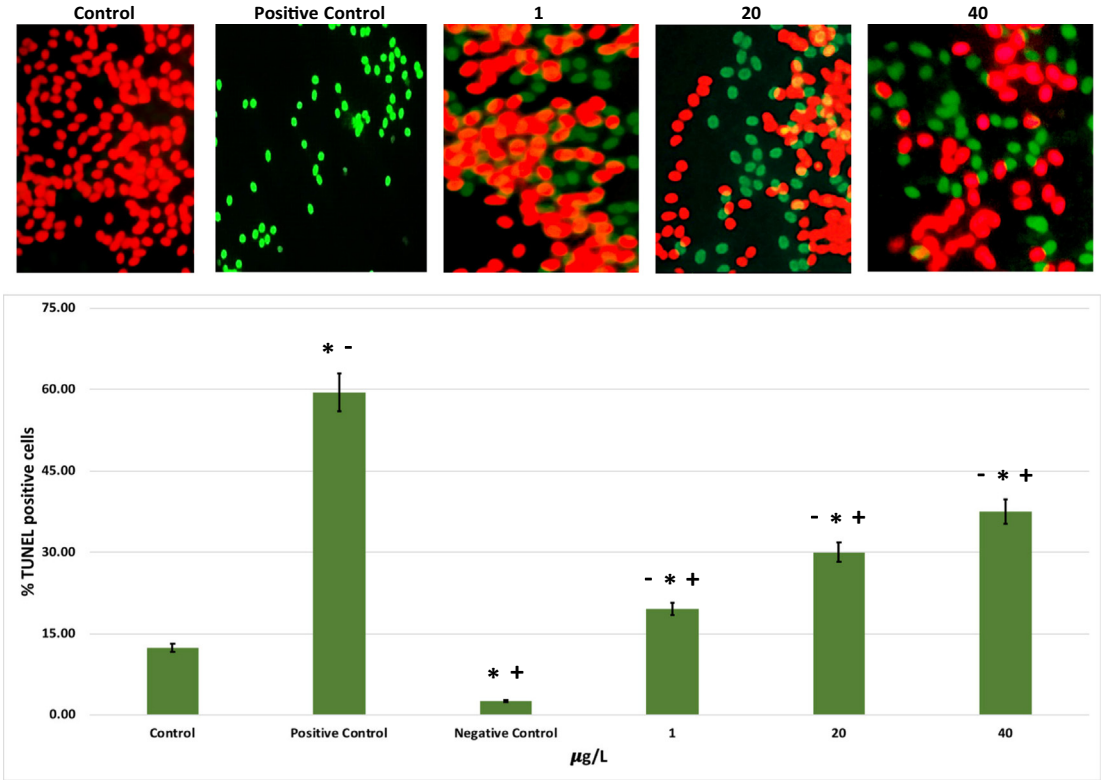


Fig. 7. Percentage of apoptotic cells in blood of *D. rerio* adults exposed to MET. Data were represented as mean ± SD, $n = 3$. * denotes a significant change in comparison to the control group. + denotes a significant change in comparison to the positive control. - denotes a significant change in comparison to the negative control.

Table 4
Concentrations of MET in the brain of zebrafish.

Nominal concentration of MET	Measured concentrations of MET in the brain of zebrafish
Control	<LOQ
1 µg/L	14 ± 1.2 ng/L
20 µg/L	274 ± 7.8 ng/L
40 µg/L	585 ± 9.2 ng/L

LOQ: limit of quantification (2 ng/L). LOD: limit of detection (10 ng/L). Data were represented as mean ± SD, $n = 3$.

AChE activity in a dose-dependent manner via oxidation of methionine, cysteine, tryptophan related to the active center. Finally, in a more recent study, Garcimartín et al., 2017 demonstrated that H_2O_2 reduced the total AChE content by modifying its isoform profile. ROS inhibition of AChE leads to physiologic abnormalities extending from behavioral damage to death (Tilton et al., 2011). Ren et al., 2015 for instance, pointed out that AChE activity inhibition might produce convulsions, paralysis, loss of coordination, and other kinds of behavioral changes in organisms.

Recently, in vitro, in vivo, and ex vivo studies have demonstrated that MET favors β–amyloid precursor protein (*APP*) and presenilin increase and induces β–amyloid (Aβ) production and aggregation (Picone et al.,

Table 5
Concentrations of MET in water samples.

	Nominal concentration	Weeks							
		1	2	3	4	5	6	7	8
Control	ND	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Atrazine	3 µg/L	2.42 ± 0.28	2.91 ± 0.30	2.40 ± 0.29	2.44 ± 0.38	2.49 ± 0.31	2.45 ± 0.29	2.41 ± 0.40	2.92 ± 0.33
	10 µg/L	8.23 ± 0.59	9.89 ± 0.63	8.18 ± 0.58	8.08 ± 0.61	8.15 ± 0.55	8.29 ± 0.64	8.31 ± 0.66	9.92 ± 0.57
MET	1 µg/L	0.82 ± 0.15	0.92 ± 0.13	0.81 ± 0.11	0.82 ± 0.12	0.80 ± 0.13	0.81 ± 0.13	0.83 ± 0.14	0.91 ± 0.12
	20 µg/L	17.30 ± 0.89	19.12 ± 0.93	17.10 ± 0.91	17.42 ± 0.88	17.17 ± 0.92	17.32 ± 0.94	16.99 ± 0.95	19.33 ±
	40 µg/L	33.11 ± 1.13	39.22 ± 1.17	32.93 ± 1.23	33.15 ± 1.19	33.29 ± 1.25	32.88 ± 1.11	33.21 ± 1.13	39.35 ±
	Nominal concentration	Weeks							
		9	10	11	12	13	14	15	16
Control	ND	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Atrazine	3 µg/L	2.43 ± 0.3	2.41 ± 0.1	2.40 ± 0.2	2.90 ± 0.3	2.43 ± 0.2	2.45 ± 0.4	2.91 ± 0.3	2.49 ± 0.4
	10 µg/L	8.30 ± 0.55	8.19 ± 0.60	8.24 ± 0.57	9.88 ± 0.59	8.20 ± 0.63	8.23 ± 0.67	9.90 ± 0.59	8.25 ± 0.64
MET	1 µg/L	0.81 ± 0.13	0.80 ± 0.12	0.82 ± 0.13	0.90 ± 0.14	0.81 ± 0.12	0.82 ± 0.14	0.93 ± 0.13	0.80 ± 0.13
	20 µg/L	17.02 ± 0.94	17.24 ± 0.90	17.13 ± 0.89	19.16 ± 0.91	17.07 ± 0.92	17.37 ± 0.98	19.22 ± 0.92	17.16 ± 0.93
	40 µg/L	33.34 ± 1.15	32.86 ± 1.19	33.08 ± 1.24	39.41 ± 1.18	33.29 ± 1.22	32.82 ± 1.17	39.07 ± 1.20	33.44 ± 1.21

LOQ: limit of quantification (1 ng/L). LOD: limit of detection (8 ng/L). Data were represented as mean ± SD, $n = 3$.

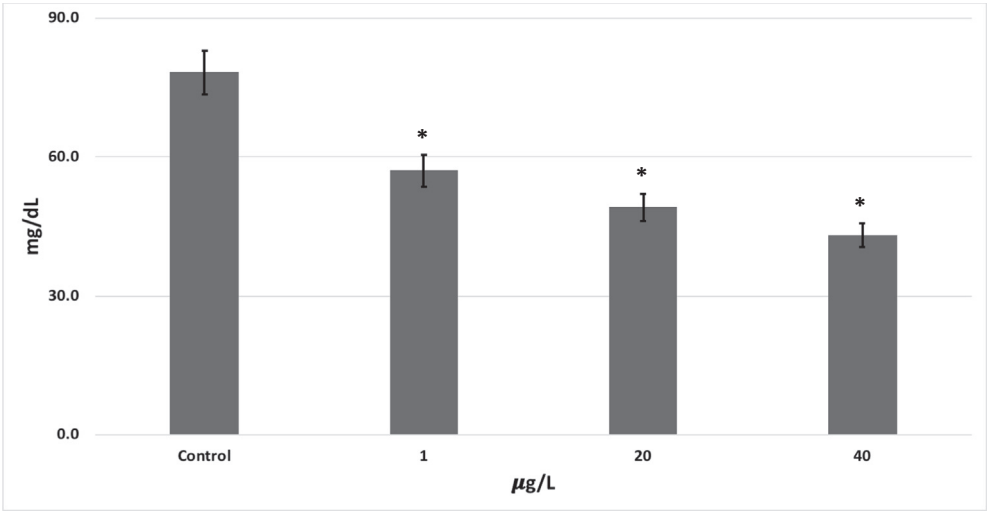


Fig. 8. Glucose blood levels of *D. rerio* adults exposed to MET. Data were represented as mean ± SD, $n = 320$. * denotes a significant change in comparison to the control group.

2015; Picone et al., 2016). In our study, we demonstrated MET at environmentally relevant concentrations (1, 20, and 40 μg/L) induces the expression of *BACE1*, *APP*, and *PSEN1* in the brain of zebrafish. Our results are in agreement with other previous studies. Chen et al., 2009, for instance, demonstrated MET, at doses that activate the AMPK signaling pathway, increased the generation of both intracellular and extracellular Aβ species. Moreover, they suggested Aβ generation is mediated by transcriptional up-regulation of *BACE1*. Nonetheless, Picone et al., 2015 and Picone et al., 2016 indicated ROS generated by MET activates the *NF-KB* transcription factor, which then translocate to the nucleus to induce the expression of *APP* and *PSEN1*. Thus, oxidative damage up-regulates Aβ via *NF-KB* transcription signaling. The over-production and aberrant self-assembly of the amyloid β peptide into fibrillar aggregates is related to neurotoxicity (Karran et al., 2011). Even though the mechanism of neurotoxicity has not been completely understood, it has been established that amyloid oligomers interact with and affect cell plasma structures by forming pores and accordingly interrupting neuronal processes (Bucciantini et al., 2012; Mannini et al., 2014; Bucciantini et al., 2014).

Besides AChE alterations in the brain and overexpression of B-amyloid-related genes, we demonstrated that MET induced the gene expression of BAX and p53 in the brain of zebrafish. In agreement with these results, Li et al., 2015 showed MET increased the protein levels of *p53* and the gene expression of *BAX* and *p21*, in a concentration-dependent manner, in MCF-7 cells. Moreover, they indicated AMPK signaling pathway is involved in *p53* upregulation. Analogously, Lin et al., 2020 demonstrated MET up-regulated the expression of *p53*, in a concentration-dependent manner, in

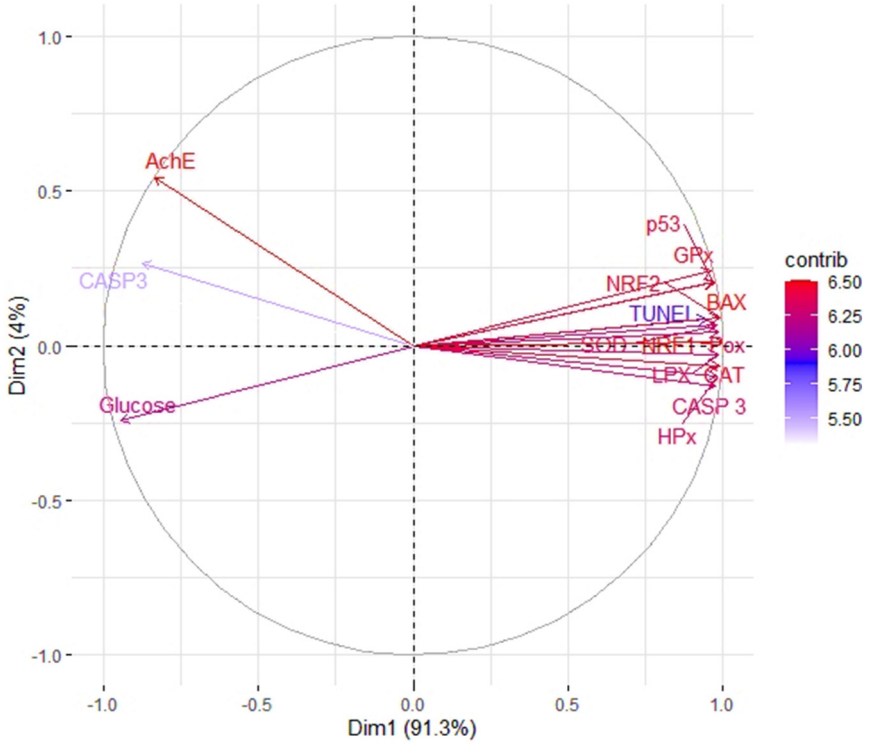


Fig. 9. Principal component analysis of *Danio rerio* biomarkers evaluated after exposure to MET.

the liver, gut, and gills of zebrafish. Concerning *BAX* and *Bcl2*, Sharma and Kumar, 2018 indicated MET upregulated the expression of *BAX* and downregulated the transcription of *Bcl2* in MCF-7, MDA-MB-231, and T47D cells. Nonetheless, in disagreement with these results, Mohammad Alizadeh et al., 2018 indicated MET downregulated *BAX* and upregulated *Bcl2* in PC12 cells. To confirm MET may induce apoptosis in *D. rerio* cells, we assessed the enzymatic activity of *CASP3* and performed a TUNEL assay. According to our results, MET did not upregulate *CASP3* and *CAS9*, but it significantly induced the activity of this enzyme, which was confirmed by a high number of apoptotic cells in the TUNEL assay. In agreement with our results, Wang et al., 2008 and Koagouw et al., 2021 demonstrated MET induces apoptosis in pancreatic cells and the gonads of *Mytilus edulis*. Since CASP3 and CASP9 were not overexpressed in fish after MET exposure, but these showed significant activity in blood samples, we believe ROS generated by MET may be involved in the activation of both enzymes. For example, it is evidence ROS causes the oxidation of cardiolipin, triggering cytochrome *c* release into the cytosol (Redza-Dutordoir and Averill-Bates, 2016). Once in the cytosol, cytochrome *c* then forms an apoptosome complex with procaspase-9, leading to CASP9 activation, which in turn activates effector caspases such as CASP3. Thus, MET may induce cell death either via upregulation of *p53* or through the overproduction of ROS species, which activate the enzyme *CASP3*.

Another function of MET is to reduce the glucose levels in organisms via inhibition of HK1 and HK2 (Marini et al., 2013; Picone et al., 2015; DeWaal et al., 2018). The above is noteworthy as several previous studies have shown low glucose levels impair brain function (Gong et al., 2006; Cryer, 2007; Mergenthaler et al., 2013; Butterfield and Halliwell, 2019; McCrimmon, 2021). Gong et al., 2006, for instance, demonstrated that impaired glucose metabolism led to neurofibrillary degeneration through a decrease in tau O-GlcNAcylation. Moreover, Cryer, 2007 pointed out prolonged hypoglycemia can cause brain death through different mechanisms such as glutamate release and activation of neuronal glutamate receptors, production of reactive oxygen species, neuronal zinc release, activation of poly(ADP-ribose) polymerase, and mitochondrial permeability transition. Therefore, the altered glucose levels may be related to the behavioral changes, the increased production of ROS, and the high number of cells death in fish exposed to MET.

5. Conclusions

Overall, this study provides new insights into the toxic effects of MET, an emerging contaminant, which consumption has increased in the last few years and led to its spilling out in high concentrations into the aquatic environment. After four months of exposure, our findings demonstrated that MET was able to cross the blood-brain barrier of zebrafish, triggering an important toxic response in the brain of these freshwater organisms. MET-exposed fish exhibited less swimming activity when compared to control fish. Additionally, chronic exposure to MET induced an important increase in oxidative damage biomarkers (LPX, HPX, and POX) and a significant decrease in AChE activity in the brain of *D. rerio* adults. Concerning gene expression, MET significantly upregulated the expression of *Nrf1*, *Nrf2*, *BAX*, *p53*, *BACE1*, *APP*, *PSEN1*, *PRKAA1*, and *PRKAA2* and downregulated *CASP3* and *CASP9*. Intriguingly, though MET did not overexpress the CASP3 gene, we saw a significant increase in the activity of this enzyme in the blood of fish exposed to MET compared to the control group, which we then confirmed by a high number of apoptotic cells in the TUNEL assay. To the best of our understanding, this is the first study that delivers evidence of oxidative impairment, apoptosis, AChE alteration, and overexpression of B-amyloid-related genes in the brain of fish exposed to MET.

CRediT authorship contribution statement

Gustavo Axel Elizalde-Velázquez, Karina Rosales-Pérez and José Manuel Orozco-Hernández performed all the exposure experiments.

Leobardo Manuel Gómez-Oliván and Gustavo Axel Elizalde-Velázquez were involved in the conception.

Leobardo Manuel Gómez-Oliván, Gustavo Axel Elizalde-Velázquez, Sandra García-Medina and Hariz Islas Flores were involved in the design and interpretation of the data and the writing of the manuscript with input from Marcela Galar-Martínez and María Dolores Hernández-Navarro.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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